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# BIOLOGICAL DELIGNIFICATION OF $^{14}$C-LABELED LIGNOCELLULOSES BY BASIDIOMYCETES: DEGRADATION AND SOLUBILIZATION OF THE LIGNIN AND CELLULOSE COMPONENTS

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ABSTRACT

Three basidiomycetes, NRRL 6464, *Pleurotus ostreatus*, and *Phanerochaete chrysosporium*, were evaluated for their ability to both solubilize and metabolize to $CO_2$ differentially $^{14}$C-labeled, naturally complexed lignocellulosics (LC). The fungi were grown on either $^{14}$C-lignin (L*C) or $^{14}$C-glucan (LC*) substrates for 20 da at 26 C. Several nutrient nitrogen (N) levels were employed to determine if this variable could influence these fungi to preferentially degrade the lignin or glucan component of LC. *Pleurotus ostreatus* and *P. chrysosporium* degraded 20% of the L*C component, whereas NRRL 6464 converted 40% of the $^{14}$C-lignin component to $^{14}CO_2$ at 2 mM N or less. At 20 mM N, lignin degradation was suppressed 15 and 60% for *P. ostreatus* and *P. chrysosporium*, respectively, after 20 da growth. NRRL 6464 was relatively unaffected by the N levels. With all three fungi, increasing levels of N yielded progressively higher $^{14}CO_2$ evolution from the LC* substrates.

Key Words: biological delignification, Basidiomycetes, lignin solubilization.

Agricultural residues contain substantial amounts of cellulose that potentially could serve as a growth substrate for either ruminants or microorganisms. However, due to the intimate association of the cellulose with other biopolymers (hemicellulose and lignin), it is not readily available as a carbon source unless the lignin component of the residues is chemically and/or biologically modified or removed. Chemical delignification has the advantage that it is a rapid process, but it is expensive and poses a potential pollution problem. Therefore, microbial delignification is gaining attention as a possible alternative to the chemical methods currently in use (Kirk *et al.*, **1978a**, **1979**).

To utilize microorganisms for delignification purposes, it is imperative to study the physiological factors that control lignolytic activity in relation to an organism's ability to metabolize cellulose. The lack of knowledge in this area results from the complex, heterogeneous structure of the lignin polymer and insensitive analytical procedures for quantifying lignin. Recently, a rather sensitive assay for measuring lignin degradation/metabolism has been developed. This assay is based upon the decomposition to $^{14}CO_2$ of either synthetic $^{14}$C-lignin (DHP) (Kirk *et al.*, **1975**) or naturally-labeled $^{14}$C-plant lignocellulosics (Crawford *et al.*, **1977**). The DHP's and $^{14}$C-plant lignocellulosics have been used effectively to study lignin degradation by white-rot fungi (Kirk *et al.*, **1976**), soil microflora (Crawford *et al.*, **1977**), and a *Nocardia* sp. (Trojanowski *et al.*, **1977**).

Studies utilizing DHP as the substrate for the lignin-degrading enzymes of the wood white-rot fungus *Phanerochaete chrysosporium* Burds. (Kirk *et al.*, **1978b**) indicate that the lignolytic activity is repressed by high levels of nutrient nitrogen (N) and derepressed by the exhaustion of nutrient N from the environment. Reid (**1979**) has also shown that the *P. chrysosporium* lignolytic activity is inhibited

[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

by high levels of nutrient N when the fungus is grown on naturally $^{14}C$-labeled lignocellulosics. This work suggests that to achieve maximal biological metabolism with minimal carbohydrate removal, N supplies should be limited while maintaining adequate levels of $SO_4^=$, $PO_4^=$, and other nutrients.

However, the C/N ratio in plant residues varies from greater than 200:1 in wood to as low as 10:1 in herbaceous plants (Levi and Cowling, **1969**; Wicklow and Carrol, **1981**). The response to N levels of organisms isolated from substrates having such diverse C/N ratios might not be the same. Recently, Wicklow *et al.* (**1980**) isolated from aged cattle dung a basidiomycete, NRRL 6464, that is relatively efficient at degrading lignin. Experiments described herein examine the effects of various nutrient N concentrations on the ability of two wood white-rot fungi, *P. chrysosporium* and *Pleurotus ostreatus* (Jacquin ex Fr.) Cummer, and a coprophilous fungus, NRRL 6464, to degrade the lignin and glucan portions of lignocellulose.

## MATERIALS AND METHODS

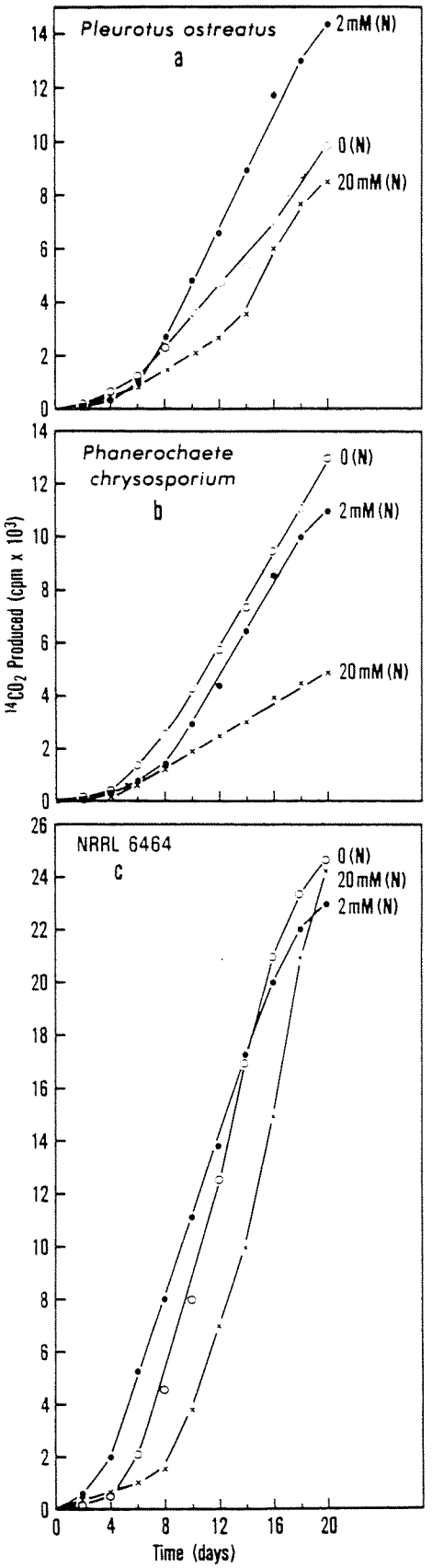
*Organisms and chemicals.*—The fungi evaluated in this study were NRRL 6464, *Phanerochaete chrysosporium* NRRL 6370 and *Pleurotus ostreatus* NRRL 2366. NRRL 6464 was originally isolated by Wicklow *et al.* (**1980**) from aged cattle dung (>6 months) collected from a pasture near Hickory Corners, Michigan. This isolate was never identified due to the immature nature of the field-collected basidiocarp and attempts to obtain fruiting bodies in culture were unsuccessful. However, of the various coprophilous fungi which successionally colonize cattle dung, this isolate degraded lignin most efficiently. We chose to include this isolate in this study because unlike the other two fungi, this organism was isolated from a graminaceous residue. The cultures were maintained upon agar slants in the ARS Culture Collection.

L-(U-$^{14}C$)-phenylalanine and D-($^{14}C$-U) glucose were purchased from New England Nuclear Corp., Boston, Mass.

*Preparation of labeled lignocellulosics.*—The lignin and cellulose components of lignocellulose in red maple (*Acer rubrum* L.) were selectively labeled with $^{14}C$ by feeding twigs an aqueous solution of either L-(U-$^{14}C$)-phenylalanine (50 $\mu$Ci/twig) or D-($^{14}C$-U)-glucose (50 $\mu$Ci/twig) through the cut stems (Crawford *et al.*, **1977**). The most recently formed xylem of the labeled twigs was excised. After the twigs were dried, ground, and the lignocellulose residues extracted, their specific radioactivities were determined by liquid scintillation counting of the $^{14}CO_2$ produced by oxidative combustion of samples. The lignin-labeled lignocellulose (L*C) contained $6.9 \times 10^3$ disintegrations per minute per mg (dpm/mg) and the cellulose-labeled lignocellulose (LC*) contained $1.5 \times 10^4$ dpm/mg.

*Fungal decomposition of differentially labeled lignocellulose.*—Ten mg of either L*C or LC* was added to 0.25 g of ground wheat straw, pelletized (cylinder = 13 mm diam), sterilized in 20 ml serum-stoppered bottles with 5 ml of media, and inoculated as previously described (Detroy *et al.*, **1980**). The experimental media were variations of a synthetic media [56 mM glucose, 14 mM $KH_2PO_4$, 2 mM

FIG. 1. Time course of $^{14}CO_2$ produced from [$^{14}C$]-lignin-labeled lignocellulose (L*C) by *Pleurotus ostreatus* (a), *Phanerochaete chrysosporium* (b), and NRRL 6464 (c). Cultures received 10 mg of the specifically labeled L*C, 56 mM glucose, and either 0 mM (○—○), 2 mM (●—●), or 20 mM (×—×) $(NH_4)_2SO_4$.


Pleurotus ostreatus
a
2mM (N)
0 (N)
20mM (N)
Phanerochaete chrysosporium
b
0 (N)
2mM (N)
20mM (N)
NRRL 6464
c
0 (N)
20mM (N)
2mM (N)
$^{14}CO_2$ Produced (cpm x $10^3$)
Time (days)

$MgSO_4 \cdot 7H_2O$, 0.5 mM $CaCl_2$ supplemented with either 0, 2, or 20 mM $(NH_4)_2SO_4$] described previously (Kirk *et al.*, **1978b**). All media were adjusted to pH 4.6. Bottles were flushed for 45 min with sterile $O_2$ every other day from the date of inoculation. All exit gases were passed continuously through scintillation vials containing $CO_2$-trapping/counting fluid, and trapped $^{14}CO_2$ present was quantified by liquid scintillation techniques as previously described (Crawford *et al.*, **1977**). Biodegradation assay of labeled substrate was followed by monitoring the percentage of total $^{14}C$ evolved as $^{14}CO_2$ from duplicate flasks during each sampling interval.

The extent of $^{14}C$-lignocellulose solubilization was measured by subjecting the aqueous phases from the reaction serum bottles to further analysis. The aqueous phases were filtered through 0.45 $\mu$m nitrocellulose filters and 0.2 ml aliquots counted by liquid scintillation techniques. The $^{14}C$-soluble counts are depicted as a percentage of the total initial $^{14}C$ radioactivity. All results represent average values obtained from duplicate fermentations. Uninoculated controls did not evolve $^{14}CO_2$ or solubilize $^{14}C$-lignocellulosics.

## RESULTS

*Effect of nutrient nitrogen on $^{14}C$-lignin-labeled lignocellulose (L*C) degradation.*—FIGURE 1 shows the time course of $^{14}CO_2$ produced from L*C under various combinations of nutrient N in the media for *P. ostreatus*, *P. chrysosporium*, and NRRL 6464. The fraction of lignin $^{14}C$-carbon converted to $^{14}CO_2$ after 20 da at 2 mM N for *P. ostreatus* was 20% as shown in FIG. 1a. Conversion of the L*C substrate to $^{14}CO_2$ at 0 and 20 mM N was 14 and 12%, respectively. Regardless of the N level, *Pleurotus* demonstrated a lag period of 4–6 da before maximal rates of $^{14}CO_2$ were produced.

FIGURE 1b depicts the time course of $^{14}CO_2$ produced by *P. chrysosporium*. At N levels of 0 and 2 mM N, *P. chrysosporium* converted 18 and 15% of the L*C carbon to $^{14}CO_2$ after 20 da. Metabolism of L*C to $^{14}CO_2$ with 20 mM N was 2.6-fold less than that produced with 0 mM N, i.e., 6.8%. As with *P. ostreatus*, a lag period of 4–8 da was observed prior to maximal rates of $^{14}CO_2$ production. These results are consistent with those presented by Reid (**1979**) and Kirk *et al.* (**1978b**, **1979**), who showed that lignin metabolism by *P. chrysosporium* is substantially higher in N-limited medium.

NRRL 6464 demonstrated a different response to N levels than either *Pleurotus* or *Phanerochaete*, in that at 20 da it metabolized the same amount of L*C regardless of the N level. This culture converted approximately 35% of the L*C to $^{14}CO_2$ in 20 da at all three N levels tested. However, NRRL 6464 was also effected by N levels. As the N levels increased, the lag periods prior to maximal rates of $^{14}CO_2$ conversion were 6, 4, and 8 da, respectively. Once the initial lag period was overcome, the culture exposed to 20 mM N showed the fastest rate of lignin conversion. This was in contrast to the N effect on *Pleurotus* and *Phanerochaete*, which demonstrated a minimal $^{14}CO_2$ evolution rate in 20 mM N. NRRL 6464 (2 mM N) was capable of metabolizing in 12 da an amount of lignin equivalent to that metabolized by the other fungi in 20 da. By the end of the

FIG. 2. Time course of $^{14}CO_2$ produced from [$^{14}C$]-cellulose-labeled lignocellulose (LC*) by *Pleurotus ostreatus* (a), *Phanerochaete chrysosporium* (b), and NRRL 6464 (c). Cultures received 10 mg of the specifically labeled LC*, 56 mM glucose, and either 0 mM (○—○), 2 mM (●—●), or 20 mM (×—×) $(NH_4)_2SO_4$.

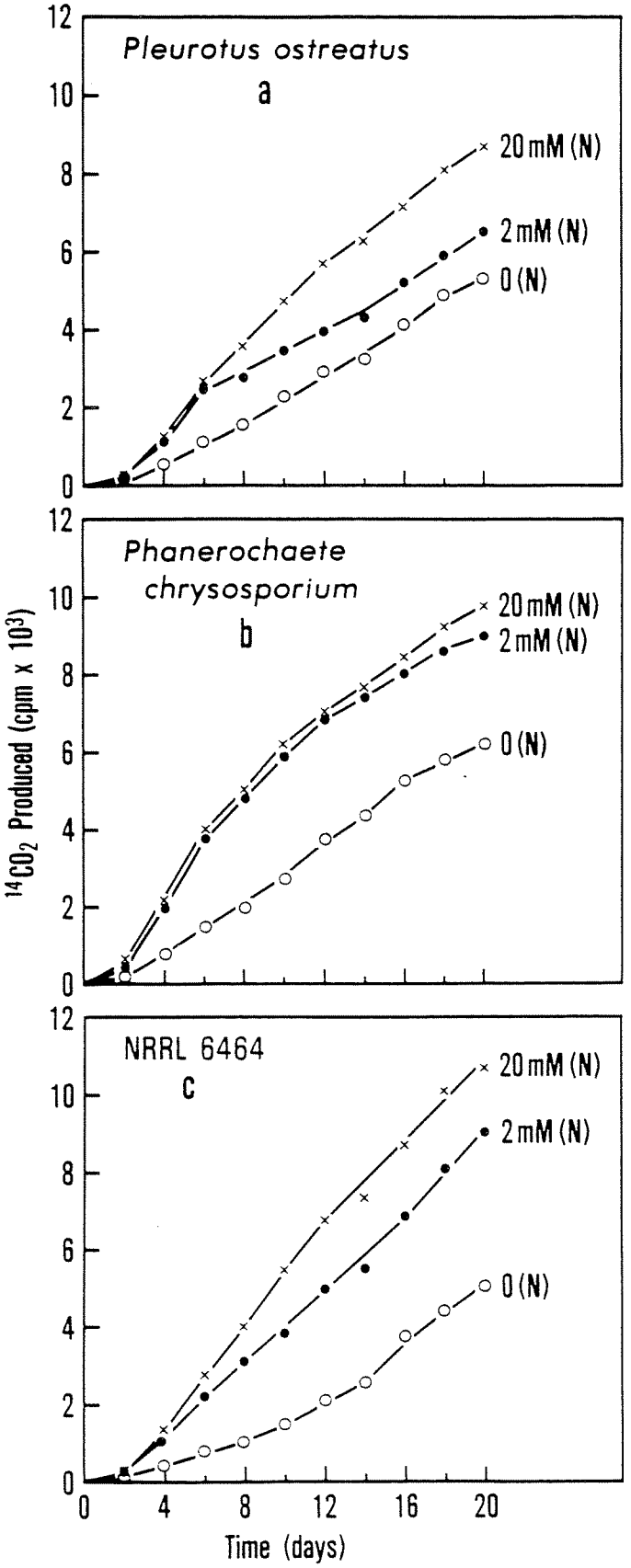

Pleurotus ostreatus
a
20 mM (N)
2 mM (N)
0 (N)
Phanerochaete chrysosporium
b
20 mM (N)
2 mM (N)
0 (N)
NRRL 6464
c
20 mM (N)
2 mM (N)
0 (N)
$^{14}CO_2$ Produced (cpm x $10^3$)
Time (days)
0
2
4
6
8
10
12
16
20

TABLE I

EFFECT OF EXOGENOUS N LEVELS UPON $^{14}CO_2$ PRODUCTION AND $^{14}C$ SOLUBILIZATION OF $^{14}C$-L*C[a] BY SOME BASIDIOMYCETES

| Organism[e] | N (mM) | $^{14}C$-soluble fraction[b] (dpm) | (%)[c] | $^{14}CO_2$ produced[d] (dpm) | $^{14}CO_2$ – $^{14}C$-soluble fraction[b] (dpm) | (%)[c] |
|---|---|---|---|---|---|---|
| Po | 0 | 34,500 | 50.0 | 9725 | 44,225 | 64.1 |
| | 2 | 9980 | 14.5 | 13,980 | 23,960 | 34.7 |
| | 20 | 8190 | 11.9 | 8315 | 16,505 | 23.9 |
| Pc | 0 | 31,760 | 46.0 | 12,470 | 44,230 | 64.1 |
| | 2 | 10,900 | 15.8 | 10,550 | 21,460 | 31.1 |
| | 20 | 3385 | 4.9 | 4720 | 8105 | 11.7 |
| NRRL 6464 | 0 | 41,870 | 60.7 | 24,500 | 66,370 | 96.2 |
| | 2 | 31,100 | 45.1 | 22,640 | 53,740 | 77.9 |
| | 20 | 33,040 | 47.9 | 24,500 | 57,540 | 83.4 |

[a] $^{14}C$-lignin lignocellulose.
[b] At 20 da.
[c] Expressed as per cent of total disintegrations per minute (dpm).
[d] Total $^{14}CO_2$ produced through 20 da.
[e] Po = *Pleurotus ostreatus*, Pc = *Phanerochaete chrysosporium*.

experiment, NRRL 6464 metabolized almost twice as much L*C as the other fungi.

*Effect of nutrient nitrogen on $^{14}C$-cellulose-labeled lignocellulose (LC*) degradation.*—FIGURE 2 depicts the time course of $^{14}CO_2$ metabolized from LC* with various nutrient N levels in the media. All three fungi produced maximal $^{14}CO_2$ at 20 da incubation in the presence of 20 mM N and minimal $^{14}CO_2$ in the absence of N. For each N level tested, the three fungi metabolized approximately the same total amount of LC* in 20 da. These results contrast those obtained using L*C as substrate (FIG. 1).

In all cases, a greater proportion of the lignin than the cellulose was metabolized to $^{14}CO_2$ by the organisms. For example, NRRL 6464 converted 35% of the lignin to $^{14}CO_2$ but converted only 7% of the LC* to $^{14}CO_2$. However, the low LC* conversion values could be a reflection of the presence of 56 mM glucose in the media, as the fungi would probably utilize the free glucose preferentially to cellulose. Also, the amount of LC degraded and incorporated into cell mass is unknown. However, it is likely that more of the carbon from cellulose than lignin would be incorporated into cell mass.

*Effect of nutrient nitrogen on $^{14}CO_2$ production and $^{14}C$ solubilization of $^{14}C$-LC.*—As a result of growth on $^{14}C$-lignocelluloses, not only was $^{14}C$ oxidized to $^{14}CO_2$, but a substantial amount of radioactivity was solubilized in the process. Culture supernatants were examined for radioactivity at the time of harvest to determine the quantity of insoluble lignin or cellulose solubilized as a result of growth by each culture.

The amount of $^{14}CO_2$ produced and $^{14}C$ solubilized by the three fungi when grown on the L*C substrate is given in TABLE I. With no added exogenous N, *Pleurotus ostreatus* solubilized 50% of the total radioactivity after 20 da. In the presence of 2 mM N, the total amount of radioactivity solubilized decreased 3.5-fold (14.5%). The amount of $^{14}CO_2$ produced, however, increased slightly. In the presence of 20 mM N, both the amount of $^{14}CO_2$ produced and $^{14}C$ solubilized decreased slightly.

TABLE II

EFFECTS OF EXOGENOUS N LEVELS UPON $^{14}CO_2$ PRODUCTION AND $^{14}C$ SOLUBILIZATION OF $^{14}C$-LC*[a] BY SOME BASIDIOMYCETES

| Organism[c] | N (mM) | $^{14}C$-soluble fraction[b] (dpm) | (%)[c] | $^{14}CO_2$ generated[c] (dpm) | $^{14}CO_2$ + $^{14}C$-soluble fraction[b] (dpm) | (%)[c] |
|---|---|---|---|---|---|---|
| Po | 0 | 4338 | 2.9 | 5750 | 10,088 | 6.7 |
| | 2 | 3731 | 2.5 | 6730 | 10,461 | 7.0 |
| | 20 | 4913 | 3.3 | 8277 | 13,190 | 8.8 |
| Pc | 0 | 7355 | 4.9 | 6145 | 13,500 | 9.0 |
| | 2 | 3641 | 2.4 | 9632 | 13,273 | 8.8 |
| | 20 | 3029 | 2.0 | 8895 | 11,929 | 7.9 |
| NRRL 6464 | 0 | 6627 | 4.4 | 5330 | 11,957 | 8.0 |
| | 2 | 6638 | 4.4 | 8290 | 14,925 | 9.9 |
| | 20 | 7687 | 5.1 | 10,770 | 18,457 | 12.3 |

[a] $^{14}C$-glucan lignocellulose.
[b–c] See TABLE I.

Of the three fungi tested, *P. chrysosporium* was the most sensitive to the addition of exogenous N. In the absence of additional N, *P. chrysosporium* solubilized 46% of the L*C. The addition of 2 mM N repressed the amount of $^{14}C$ solubilized threefold while inhibiting the amount of $^{14}CO_2$ produced by about 15%. In the presence of 20 mM N, only 4.9% of the L*C was solubilized and only 6.8% metabolized to $^{14}CO_2$.

In contrast, NRRL 6464 was relatively unaffected by the various nutrient N levels employed. The maximal amounts of lignin solubilized and metabolized occurred in the absence of exogenous N. However, the amount of $^{14}CO_2$ produced in the presence of N was equivalent in all cases. The addition of 2 mM or 20 mM N reduced the amount of $^{14}C$-lignin solubilized by only 25%.

When grown on LC*, the cultures solubilized 2.9–5.1% of the total LC* available (TABLE II). The various N levels employed had virtually no effect upon the amount of LC* solubilized by *P. ostreatus* and NRRL 6464. *Phanerochaete chrysosporium* did, however, appear to solubilize slightly less LC* with increasing N levels. In general, all three fungi metabolized slightly more LC* as the N level was increased. The three fungi metabolized ($^{14}CO_2$) and solubilized a maximum of 8.8–12.3% of the LC* substrate. In contrast, these cultures degraded between 64 and 96% of the L*C substrate (TABLE I).

## DISCUSSION

From the results, two important points should be noted. First, if biodelignification is measured only by the quantity of $^{14}CO_2$ produced during the course of the fermentation, only a minimum, and potentially misleading, estimate of lignin degradation is obtained. For example, using only the $^{14}CO_2$ data, our results indicated that both *Pleurotus ostreatus* and NRRL 6464 were relatively tolerant of high nutrient N levels. However, when the solubilization data were included in the analysis, it was clear that lignin degradation by *P. ostreatus* was dramatically repressed by high N levels. Thus, the $^{14}CO_2$ measurement is not really a true measure of lignin degradation, but rather a measure of an organism's ability to metabolize lignin or lignin breakdown products to $CO_2$.

Secondly, our results suggest, but by no means prove, that the mechanisms by which an organism solubilizes lignin and metabolizes lignin to $CO_2$ are differ-

ent. One would expect N to repress both systems equally if these processes were performed via a single mechanism. This was not the case. The results with *P. chrysosporium* show that 2 mM N causes a 300% decrease in amount of $^{14}$C-lignin solubilized but only a 15% decrease in the amount of $^{14}CO_2$ produced.

Our results on the effect of nutrient N on lignin degradation by *P. chrysosporium* are similar to those reported by Kirk *et al.* (**1976**, **1978a**), Reid (**1979**), and Yang *et al.* (**1979**). *Pleurotus ostreatus* demonstrated a N repression pattern similar to that of *P. chrysosporium*, in that high N levels repressed the organisms' ability to degrade lignin. NRRL 6464 showed a different response to nutrient N levels in that its ability to degrade and solubilize lignin was virtually unaffected by the various N levels tested.

A possible explanation for the different responses to nutrient N shown by *P. chrysosporium* and *P. ostreatus*, as compared to NRRL 6464, may lie in the environment from which these organisms were isolated. Both *Phanerochaete* and *Pleurotus* are typical wood white-rot fungi, whereas NRRL 6464 was originally isolated from cattle dung (Wicklow *et al.*, **1980**). The C/N ratios of wood (200:1; Levi and Cowling, **1969**) and cattle feces (16 to 26:1; D. T. Wicklow, personal communication) vary dramatically. Recently, Wicklow *et al.* (**1980**) argued that Basidiomycetes colonizing ruminant dung in grassland ecosystems represent the ecological equivalents of wood white-rot fungi. This report supports this hypothesis in that the organisms typically isolated from substrates with limited N degrade lignin best under limited N environments, and the organism isolated from a relatively high N environment was essentially unaffected by N. The available N in plant residues appears to play an important role as to whether certain white-rot fungi are able to degrade the lignin polymers in different residues. This suggests that one particular organism may not efficiently degrade lignin in all residues, but rather, depending upon the chemical nature of the residue, different microbes might have to be employed to effect maximal lignin degradation.

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